## REMARKS

### Status of Claims

Claims 1, 2, 5-21, and 23-30 are pending. Claims 1-2, 5-9, 20-21, 23-27 have been rejected. Claims 10-19 and 28-30 stand withdrawn. Claims 1 and 20 have been amended. Support for the amended claims can be found in paragraphs 0086, 0087, 0145, and in Figure 1 of the published Application specification. Applicants assert that no new matter has been introduced.

## CLAIM REJECTIONS

### 35 U.S.C. § 102 Rejections

In the Office Action, the Examiner rejected claims 1, 2, 5-9, 20-21, 23-27 under 35 U.S.C. § 102(a), as allegedly being anticipated by Pawelek et al. (US patent 6,685,935).

The Examiner alleged that Pawelek et al. inherently anticipates the invention since it describes the steps of A) administering to an animal a bacterial vector, B) passaging the vector through the animal, C) harvesting the bacterial vaccine vector and D) repeating steps A-C to enhance the immunogenicity of the vector and where the bacterial vaccine vector expresses a heterologous antigen. Applicants disagree.

Pawelek et al. describe methods of attenuating bacteria for its virulence (see Col. 24, lines 50-67) rather than methods of stabilizing virulence, much less achieving this stabilization after two passages with the bacterial vaccine vector, as claimed. The present invention shows that after only two passages there is an increase in activity, i.e.-virulence and enhanced immunogenicity (see Figure 1 of present invention), and that this activity remains stable throughout subsequent passages, thereby demonstrating stabilization of virulence throughout the passage cycle. Hence, this observation eliminates the need for subsequent passages and enhances the safety of the vaccine since a lower dose of vaccine can be used. Further, this decreases the risks associated with injection of bacterial debris (arising from dead bacteria) which may have unspecific toxicity for the host leading to unpredictable and adverse effects (see Peters et al. pg 1193, top left column, Vaccine 21 (2003) 1187–1194, **attached**).

Moreover, Pawelek et al. does not describe repeating steps of administering, passaging and harvesting with the harvested bacterial vector until a maximum bacterial load in organ is reached (emphasis added) as claimed, and this 'maximum bacterial load' is also indicative of the 'maximum bacterial virulence', see paragraph 0087 of the published specification. Therefore, Pawelek et al. does not describe repeating the above steps until a maximum bacterial load or maximum bacterial virulence is reached but rather discusses carrying out genetic manipulation for the attenuation of virulence (see Section 6.2.2, Col. 24, lines 8-67).

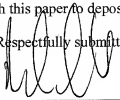
In addition, claim 2 is not anticipated by Pawelek et al. since there is no disclosure of the spleen as being one of the organs. Applicants therefore request withdrawal of the claim rejections.

In view of the foregoing remarks, Applicants assert that the pending claims are allowable. Their favorable reconsideration and allowance is respectfully requested.

Should the Examiner have any question or comment as to the form, content or entry of this Amendment, the Examiner is requested to contact the undersigned at the telephone number below. Similarly, if there are any further issues yet to be resolved to advance the prosecution of this application to issue, the Examiner is requested to telephone the undersigned counsel.

Please charge any fees associated with this paper to deposit account No. 50-3355.

Respectfully submitted,



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# Enhancing the immunogenicity of bioengineered *Listeria monocytogenes* by passaging through live animal hosts

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**Abstract**

Bioengineered *Listeria monocytogenes* can be used as a recombinant bacterial vaccine vector for the induction of strong cell-mediated immunity to passenger antigens. *Listeria* loses virulence after undergoing bioengineering techniques, thus decreasing its efficacy as a vaccine vector. We addressed this problem by examining the virulence, and the ability to induce $CD8^+$ T-cells, of *Listeria monocytogenes* vaccine strains before and after passaging through mice. We found that two in vivo passages are required to restore the induction of cell-mediated immunity to passenger antigens and maximum virulence to these strains. In addition, we found that after each passage, harvested bacteria must be cloned and checked for expression of the bioengineered gene to counter selection in favor of antigen loss mutants.



*Keywords:* *Listeria monocytogenes*; Recombinant vaccine; Virulence; Antigen loss

## 1. Introduction

The advantages that *Listeria monocytogenes* possesses as a vaccine vector are rooted in its biology. It is a beta hemolytic gram positive, facultative intracellular bacterium that has been used to study cell-mediated immunity for decades [1]. Unlike other intracellular organisms such as *Salmonella* and BCG, *Listeria* escape into the cytoplasm of the host cell by disrupting the phagosomal membrane, mainly through the action of hemolysin, also known as listeriolysin O (LLO) [2]. Because the bacteria replicate in the cytoplasm without coming into contact with the extracellular compartment, humoral immunity (neutralizing antibody) does not play a major role in resistance to listerial infections [1]. Peptides derived from *L. monocytogenes* in the phagolysosome can be presented via both the MHC class I and class II pathways, and thus induce both $CD4^+$ and strong $CD8^+$ T-cell responses. *Listeria* has received increasing attention as a vaccine vector because of these unusual characteristics and immunological properties. *L. monocytogenes* has been shown to be a potent vaccine vector for infectious and neoplastic disease [3–14].

The effectiveness of *Listeria* (or, indeed, any live, attenuated pathogen) as a vaccine vector depends on its ability to induce strong, long-lived immune responses to the passenger antigen. Bioengineering techniques tend to decrease the virulence of *Listeria*, probably because the expression of virulence factors needed for in vivo survival in the host is either turned off or reduced [15,16]. Avirulent bacteria do not, as a rule, invoke strong immune responses [17,18]. Thus, although bioengineering techniques make it possible to use *Listeria* as a vaccine vector, they may also decrease its efficacy.

The process of introducing a new gene to the bacterial genome requires extensive propagation of the bacterium in a rich medium optimized for bacterial growth in vitro [19]. In such media, bacteria do not need to express the virulence factors that they would need to survive the hostile environment of a host in an infection. Indeed, cultivated bacteria may shut down virulence factors not needed in an in vitro growth medium. For example, the expression of the essential virulence factor LLO (required for the escape of *Listeria* from the phagolysosome) is shut down in iron-rich medium [20]. When in vitro cultured bacteria are subjected to in vivo conditions, the bacteria may not be able to reactivate their virulence factors rapidly enough to avoid the host's innate immune defenses. A method to restore and enhance the virulence of bacteria for experimental purposes is to passage the organisms through animals [21]. The molecular basis for this

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method is not known, but it is thought that passaged bacteria are able to more effectively and rapidly regulate their virulence factors. In this study, we address the question whether passaging can enhance the immunogenicity of the recombinant vaccine vector *L. monocytogenes*. We determined that passaging the recombinant *Listeria* enhances not only the immunogenicity of the bacterium but also the induction of antigen-specific $CD8^+$ T-cells towards the passenger gene.

We have routinely observed a reduction in virulence of two–four logs in recombinant *L. monocytogenes* vaccines compared to wild type strains imposed by the expression of the foreign gene. Thus antigen loss variants of recombinant *L. monocytogenes* could arise in vivo due to their selective growth advantage. We also report here an example of this phenomenon and approaches to address this practical problem.

## 2. Materials and methods

### 2.1. Bacterial strains

*L. monocytogenes* strain 10403S, serotype 1, was the wild type organism used in these studies and the parental strain of the constructs described below. It has an $LD_{50}$ of approximately $5 \times 10^4$ CFU when injected i.p. into BALB/c mice. Lm-Gag refers to a recombinant strain of *L. monocytogenes*, which carries a copy of the HIV-1 strain HXB (subtype B laboratory strain with a syncitia forming phenotype) *gag* gene stably integrated into the listerial chromosome using a modified shuttle vector pKSV7 as previously described [11]. Gag protein is expressed and secreted by *L. monocytogenes* as determined by Western blot.

Lm-E7 refers to a recombinant strain of *L. monocytogenes* which carries a copy of the human papilloma virus (HPV) *E7* gene in the listerial chromosome. It was constructed similarly to Lm-Gag with some modifications. Lm-LLO-E7 is constructed as a *hly-E7* fusion gene in an episomal expression system. The construction of both E7 expressing recombinant strains is described in detail elsewhere [12]. All strains were grown in brain–heart infusion (BHI) broth or agar plates (Difco Labs, Detroit, MI).

### 2.2. Bacterial culture

To prepare bacterial pools for passaging, bacteria from a single clone expressing the passenger antigen were selected and cultured in BHI broth overnight. Aliquots of this culture were frozen at −70 °C with no additives. From this stock, cultures were grown to 0.1–0.2 O.D. at 600 nm, and aliquots were again frozen at −70 °C with no additives. To prepare cloned bacterial pools, the above procedure was used, but after each passage a number of bacterial clones were selected and checked for expression of the target antigen. Clones in which expression of the foreign antigen was confirmed were used for the next passage.

### 2.3. Passage of bacteria in mice

Female BALB/c ($H\text{-}2^d$) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained in a pathogen-free microisolator environment. Mice used in this study were 6–8 weeks old.

The titer of viable bacteria in an aliquot of stock culture, stored frozen at −70 °C, was determined by plating on BHI agar plates on thawing and prior to use. In all experiments $5 \times 10^5$ bacteria were injected intravenously into BALB/c mice. After 3 days, the spleen was harvested, homogenized, and serial dilutions of the spleen homogenate were incubated in BHI broth overnight and plated on BHI agar plates. For further passage, aliquots were again grown to 0.1–0.2 O.D., frozen at −70 °C, and the bacterial titre again determined by serial dilution. After the initial passage (passage 1), this sequence was repeated for a total of four times.

### 2.4. Western immunoblotting

Lm-E7 clones from various passages were grown overnight at 37 °C in LB broth (Difco Labs, Detroit, MI). Equivalent numbers of bacteria, as determined by O.D. at 600 nm absorbance, were pelleted and 18 ml of each supernatant was TCA precipitated. A 200 μl aliquot of the pellet was lysed by incubating with 2 mg/ml lysozyme at 37 °C for 2 h. E7 and LLO expression in the supernatant and lysed cell pellet was analyzed by Western blot. Proteins were precipitated using TCA and run in an SDS-poly-acrylamide gel as previously described [12]. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (NEN Life Sciences, Boston, MA) using a Western blot apparatus (BioRad, Melville, NY). Blots were stained with a 1:2000 dilution of either a rabbit anti-HPV E7 antiserum (kindly provided by Dr. Drew Pardoll, Johns Hopkins University, Baltimore, MD) or an anti-LLO rabbit anti-serum (kindly donated by Dr. Howard Goldfine, University of Pennsylvania). Blots were counter stained with horseradish peroxidase-labeled anti-rabbit goat Ab (Amersham, Arlington Heights, IL) and developed using ECL detection reagent kits (Amersham, Arlington Heights, IL) on Scientific Imaging Film (Kodak, Rochester, NY).

### 2.5. Polymerase chain reaction of Listeria ORF genes and passenger genes HPV E7

E7 was amplified by PCR using the primers 5′-GGCTCGAGCATGGAGATACACC-3′ and 5′-GGGGACTAGTTTATGGTTTCTGAGAACA-3′ from the respective bacterial passages that express or do not express the HPV *E7* gene as determined by Western blot. The open reading frame from *Listeria* ORF *XYZ* was amplified using the primers 5′GGGAATTCAAGCAACGGGAAGACATG -3′ and 5′-GGAAGCTTCGGCGAAGTCATCACTG-3′. Sequencing of PCR products was performed by the Children's Hospital of Philadelphia sequencing facility.

### 2.6. Intracellular cytokine stain for IFN-γ

Lymphocytes were cultured for 5 h in complete RPMI-10 medium supplemented with 50 U/ml human recombinant IL-2 and 1 μl/ml Brefeldin A (Golgistop™; PharMingen) in the presence or absence of either the cytotoxic T-cell (CTL) epitope for HIV-GAG (AMQMLKETI), *Listeria's* LLO (GYKDGNEYI) or the the HPV virus gene *E7* (RAHYNIVTF). The peptides were used at a concentration of 1 μM. The cells were first surface-stained, then washed and subjected to intracellular cytokine stain using the Cytofix/Cytoperm kit in accordance with the manufacturer's recommendations (PharMingen). For intracellular IFN-γ stain, we used FITC-conjugated rat anti mouse IFN-γ mAb (clone XMG 1.2) and its isotype control Ab (rat IgG1; both from PharMingen). In all $10^6$ cells were stained in PBS containing 1% BSA and 0.02% sodium azide (FACS™ buffer) for 30 min at 4 °C followed by three washes in FACS buffer. Sample data were acquired on either a FACScan™ flow cytometer or FACSCalibur™ instrument (Becton Dickinson, San Jose, CA).

Three-color flow cytometry for CD8 (PERCP conjugated, rat anti mouse, clone 53-6.7 Pharmingen), CD62L (APC conjugated, rat anti mouse, clone MEL-14) and intracelluar IFN-γ was performed using a FACSCalibur™ flow cytometer and data were further analyzed with CELLQuest software (Becton Dickinson, Mountain View, CA). Cells were gated on $CD8^{high}$ and $CD62L^{low}$ before they were analyzed for $CD8^+$ and intracellular IFN-γ staining.

## 3. Results

### 3.1. Passaging recombinant L. monocytogenes in mice increases its virulence

To determine the impact of passaging on recombinant *Listeria*, we used three different constructs. Two of these constructs carry a genomic insertion of the passenger antigen: the first carries the HIV *gag* gene (Lm-Gag), and the second carries the HPV *E7* gene (Lm-E7). The third (Lm-LLO-E7) carries a plasmid with the fusion gene for the passenger antigen (HPV E7) fused with a truncated version of LLO and a gene encoding PrfA, the positive regulatory factor that controls *Listeria's* virulence factors. This plasmid was used to complement a *prfA* negative mutant so that in a live host, selection pressures would favor conservation of the plasmid, because without it the bacterium is avirulent. All three constructs have been propagated extensively in vitro for many bacterial generations.

We found that bacterial virulence, as measured by numbers of surviving bacteria in the spleen, increases with each of the first two passages. With Lm-Gag and Lm-LLO-E7, we found that virulence increases with each passage up to passage 2 (Fig. 1). The plasmid-containing construct, Lm-LLO-E7, showed the most dramatic increase in virulence.

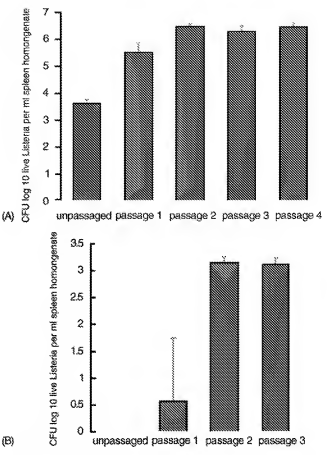


Fig. 1. Effect of passaging on bacterial load (virulence) of recombinant Lm-Gag (A) and Lm-LLO-E7 (B) in the spleen. The average CFU of live bacteria/ml of spleen homogenate from four mice is shown. This experiment was repeated at least three times with reproducible results, only one set of data is shown.

With this construct, we had to increase the immunizing dose of bacteria to $10^7$ bacteria and to harvest the spleen on day 2 in order to find bacteria in the spleen compared to an initial dose of $10^5$ bacteria for Lm-Gag harvested on day 3. After this initial passage, we were able to use the standard dosage; apparently, the initial passage had increased the virulence of the bacteria so that they were able to reproduce despite the rigors of the host environment.

### 3.2. Loss of passenger antigen gene expression can occur in vivo

As we passaged Lm-E7 for the third time, we noticed an increase of virulence of about two logs. We saw, however, that the increase in virulence from passage 2 to passage 3 was substantially greater than from unpassaged bacteria to passage 1 (Fig. 2). On further investigation, we discovered that the bacteria harvested from passage 3 had stopped expressing the passenger antigen as determined by Western blotting. (Fig. 3). By conducting PCR analysis, we found that the inserted gene coding for the passenger antigen had not been deleted, and was the same size as the expressed

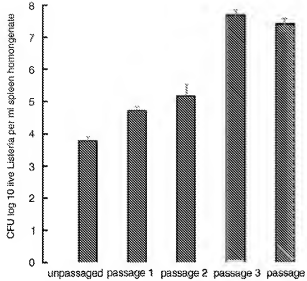


Fig. 2. Effect of passaging on bacterial load (virulence) of recombinant Lm-E7 in the spleen. The average CFU of live bacteria/ml of spleen homogenate from four mice is shown.

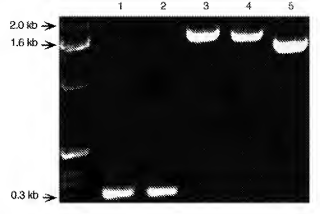


Fig. 4. PCR analyses of the nonexpressing Lm-E7 shows amplification of a normal sized *E7* gene in the listerial chromosome. Nonexpressing and expressing Lm-E7 clones were amplified by PCR. Amplification of the open reading frame does not show any alteration in gene size. Lane 1, *E7* gene Lm-E7 (passage 2); lane 2, *E7* gene Lm-E7 (passage 1); lane 3, ORF *XYZ*, Lm-E7 (passage 2); lane 4, ORF *XYZ*, Lm-E7 (passage 1); lane 5, ORF *XYZ*, Lm wild type 10403s.

gene (Fig. 4). We found no deviation from the original open reading frame, indicating that no transposon or deletion had occurred. When we sequenced the gene (Fig. 5), we found a single nucleic acid deletion mutation had occurred in position 10 at the 5′ end, leading to a frame shift that inserted multiple stop codons into the gene. This frame shift apparently halted expression of the gene. We then cloned bacteria produced by passage 1, selected a clone expressing the passenger antigen, and performed the passaging procedure again. We found that the virulence of the intact Lm-E7 increased by about 1.5 logs over unpassaged bacteria (Fig. 6).

### *3.3. Passaging increases the ability of L. monocytogenes to induce CD8+ T-cells*

We then investigated the impact of passaging on the induction of antigen-specific $CD8^+$ T-cells. For this purpose, we chose intracellular cytokine staining with immunodominant peptides specific for MHC-class I using HIV-Gag peptide AMQMLKETI [22]and LLO 91–99 (GYKDGNEYI) [23]. When we injected a dose of $10^3$ CFU passaged bacteria (Lm-Gag) into mice, we obtained a good induction of HIV-Gag-specific $CD8^+$ T-cells. The same dose of Lm-Gag that had not been passaged, however, induced no detectable level of HIV-Gag-specific $CD8^+$ T-cells. Next, we investigated whether a higher dose of unpassaged bacteria would compensate for their relative avirulence. At a 100-fold higher dose, unpassaged bacteria still did not yield any detectable induction, while the same dose increase with passaged bacteria increased the number of HIV-Gag-specific T-cells by 50% (Fig. 7).

To analyze whether the lack of induction of $CD8^+$ T-cells with unpassaged bacteria may be linked to properties of the passenger antigen, we also investigated the induction of listeriolysin-specific $CD8^+$ T-cells. LLO elicits a similar pattern of immune response: a low dose of passaged bacteria elicited a good response, while a low dose of unpassaged bacteria elicited no detectable response (a high dose

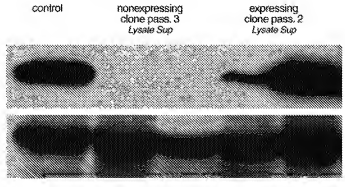


Fig. 3. Lm-E7 passaged three times without recloning does not express or secrete E7. E7 and Listeriolysin expression analyzed by Western blot in passages 2 and 3 of Lm-E7 in the whole cell lysate (Lysate) and secreted into the superantant (sup).

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  1 ATGAAAAAAATAATGCTAGTTTTTTATTACACTTATTATTAGTTAGTCTACC 50
    |||||||||| ||||||||||||||||||||||||||||||||||||||||||
  1 ATGAAAAAA-TAATGCTAGTTTTTTATTACACTTATTATTAGTTAGTCTACC 50

 51 AATTGCGCAACAAACTGAAGCAAAGGATGCATCTGCATTCAATTGGATCCC 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
 51 AATTGCGCAACAAACTGAAGCAAAGGATGCATCTGCATTCAATTGGATCCC 100

101 ATGGAGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAG 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
101 ATGGAGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAG 150

151 ACAACTGATCTCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGAGGA 200
    |||||||||||||||||||||||| ||||||||||||||||||||||||||
151 ACAACTGATCTCTACTGTTATGAGTAATTAAATGACAGCTCAGAGGAGGA 200

201 GGATGAAATAGATGGTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATT 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
201 GGATGAAATAGATGGTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATT 250

251 ACAATATTGTAACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTGTGC 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
251 ACAATATTGTAACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTGTGC 300

301 GTACAAAGCACACACGTAGACATTCGTACTTTGGAAGACCTGTTAATGGG 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
301 GTACAAAGCACACACGTAGACATTCGTACTTTGGAAGACCTGTTAATGGG 350

351 CACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCATAA       400
    ||||||||||||||||||||||||||||||||||||||||||
351 CACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCATAA       400
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Fig. 5. Nucleic acid sequence from clones that do not express HPV E7 have a frameshift mutation in position 10 of their HPV *E7* gene. Sequences of the passenger gene, *E7*, from clones expressing HPV E7 (passage 2) and the nonexpressing clones (passage 3) are shown.

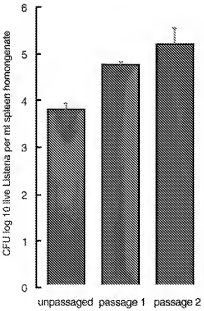


Fig. 6. Effect of passaging on bacterial load (virulence) of cloned recombinant Lm-E7 in the spleen. The average CFU of live bacteria/ml of spleen homogenate from four mice is shown. This experiment was repeated at least three times with reproducible results only one set of data is shown.

elicited a moderate response). Thus, the failure to induce antigen-specific $CD8^+$ T-cells is not restricted to the passenger antigen but also extends to LLO, an endogenous antigen of *Listeria*.

## 4. Discussion

In this study, we confronted certain practical problems arising from propagation of bioengineered *Listeria* in vitro. After genetic manipulation over many bacterial generations, the bacteria become less virulent and induce a poor cellular response. Our study aimed to determine whether passaging is a viable technique to increase the effectiveness of *Listeria* as a vaccine vector by increasing virulence and the induction of passenger antigen-specific $CD8^+$ T-cells. At the same time, we wished to determine methods for handling potentially negative effects of passaging, such as loss of protein expression through genetic mutations in the passenger antigen.

We found that passaging recombinant *Listeria* through mice does indeed cause an increase of virulence, and more to the point, an increased induction of antigen-specific $CD8^+$ T-cells. This increased induction not only permits effective

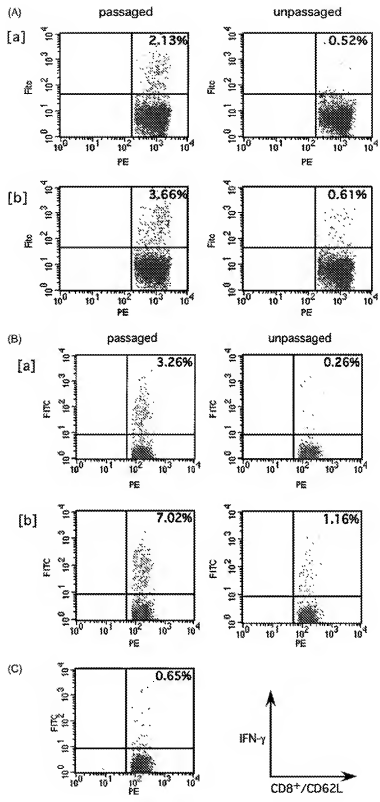


Fig. 7. Passaged Lm-Gag induces more antigen-specific $CD8^+$ T-cells for HIV-Gag and LLO than unpassaged Lm-Gag. Groups of 4 BALB/c mice were immunized with either $10^5$ CFU [a] or $10^5$ CFU [b] passaged and unpassaged Lm-Gag. After 9 days of immunization, spleens were harvested and stimulated for 5 h with the class I, HIV-Gag peptide (Panel A) or the LLO peptide (Panel B) in the presence of 50 U/ml IL-2 and Brefeldin A. Panel C shows splenocytes from mice immunized with $10^5$ CFU passaged Lm-Gag stimulated with a control peptide from HPV E7. The splenocytes were stained with anti-CD8 and anti-CD62L, fixed, permeablized and stained for intracellular IFN-γ. The populations analyzed in the figure are $CD8^+$, $CD62L^{low}$. This experiment was repeated at least three times with reproducible results only one set of data is shown.

deployment as a vaccine vector, but also confers ancillary advantages: a lower dose of vaccine can be used, thus decreasing the risks associated with injection of bacterial debris (arising from dead bacteria) which may have unspecific toxicity for the host leading to unpredictable and adverse effects. The molecular basis for the increase in virulence and induction is currently not well investigated, but we speculate that passaging brings selective pressures to bear on the bacteria, and encourages the bacteria's ability to respond more quickly with the upregulation of virulence factors to the host's defense systems [15,24]. However, it should be noted that this hypothesis is not supported by studies in rabbits where no measurable increase in the production of hemolysin and phospholipase was observed between unpassaged and passaged bacteria [21,25]. For vaccine vector purposes, the key advantage of passaging is that it increases the desired immune response. Literature in the field shows that a strong specific $CD8^+$ T-cell response is necessary to confer protection against disease on the host [26]. North et al. have shown that the strength of this response depends on bacterial load and the length of exposure [26]. Because less virulent bacterial vectors cannot survive as effectively in the inductive sites of the immune system [16,27,28], use of such vectors decreases bacterial load and length of exposure [17,18,27]. We selected the Lm-Gag construct to explore effective immune induction. Our study demonstrated that even at a relatively low dose of $10^3$ CFU (0.002 $LD_{50}$), passaged bacteria (Lm-Gag) effectively induced antigen-specific $CD8^+$ T-cells at a frequency of 2.13% (specific T-cells as a percentage of activated T-cells) for HIV-Gag and 3.26% for LLO; at the same time, the unpassaged Lm-Gag vaccine vector was unable to produce any detectable T-cells (Fig. 7A and B [a]).

We attempted to compensate for the lower levels on day 3 by increasing the initial dosage a 100-fold to $10^5$ CFU (0.2 $LD_{50}$) and analyzed the induced $CD8^+$ T-cells again. This dramatic increase in the immunizing dose increased the number of antigen-specific T-cells induced by the passaged bacteria but the unpassaged vector still produced levels of antigen-specific $CD8^+$ T-cells (Fig. 7A and B [b]) that were barely above background (Fig. 7C). This observation was not confined to the passenger antigen (Fig. 7A) but was also the case for the immunodominant Listerial antigen LLO (Fig. 7B). Thus, only passaged bacteria were able to induce a $CD8^+$ T-cell population in a dose-dependent manner. We conclude that the quality rather than the quantity of the vector determines effective induction of $CD8^+$ T-cells. We believe that *Listeria's* method of escape explains this effect. Only bacteria that escape the primary phagolysosome introduce gene products into the class I antigen processing pathway, the pathway responsible for the induction of $CD8^+$ T-cells. Bacteria that are not adapted to the hostile conditions of the innate immune system will be less able to escape from the primary phagolysosome, a defense strong enough to defeat most bacteria even in a virulent population [29].

With one of the constructs that we used, Lm-E7, in vivo passaging resulted in the emergence of a recombinant harboring a mutation that shuts down expression of the passenger antigen. These data indicate that the production and/or secretion of the HPV E7 protein is a major burden leading to a significant attenuation when compared to the construct that carries but does not express the HPV *E7* gene. The expression of the passenger gene clearly attenuates the bacterium resulting in an increase in the $LD_{50}$ of about two logs over the wild type strain (10403s $LD_{50} = 5 \times 10^4$). The mechanism by which this attenuation occurs remains unclear, and is beyond the scope of this paper. However, we have observed an attenuation of virulence in all of the recombinant vaccine strains we have produced, whether the transgene is episomally or chromosomally expressed. We believe this is caused by metabolic burdens imposed generally by the presence and expression of a eucaryotic gene in a procaryotic organism, rather than to factors specific to a particular genetic insertion.

In the context of deploying *Listeria* as a vaccine vector, loss of expression of the antigen under in vivo pressure is clearly undesirable. We found, however, that this effect could be avoided by a simple technique. After each round of passaging, we selected a number of clones and checked them for expression before using them as seed stock. This provided quality control for retention of gene expression and virulence. Thus in vivo passaging of genetically engineered bacteria in mice also provides a method of screening vaccine stocks for unstable mutants that could arise during their use in humans.

Where the bacteria have been cloned to assure retained expression, we did not observe an increase of virulence after the passaging procedure had been repeated two times (that is, the passage 2 population showed maximal virulence). In a practical setting, knowing the maximal virulence makes the effects of a given dosage easier to predict. It decreases the concern that if the bacterial vector is given to a compromised host where innate selection pressure is not very high, the bacteria could take advantage of these less stringent conditions to recover a potentially dangerous virulence in the host. To understand how this could occur, consider that a longer lag period in the host before the bacterium is able to upregulate its virulence factors usually enables the host to eliminate or control the bacterium. In a compromised host, a less potent innate immune system may not be able to use this lag period effectively. In this case, the bacterium can increase virulence to the point that it poses a threat to the host. Passaging minimizes this effect because it permits administration of the vaccine vector with maximal virulence, with a decreased potential for unforeseen changes. However, with a live bacterial vector, mutations in the host remain possible, and some such mutations may tend to approach the wild type virulence. With this in mind, we are currently developing a number of attenuated vaccine strains to determine whether such strains can provide an additional margin of safety, without unduly compromising immune effectiveness.

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